THE ROLE OF NCK ADAPTORS IN THE REGULATION OF ENDOTHELIAL CELL-CELL ADHESION

A Senior Scholars Thesis

by

KRISTYN BRAY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Approved by:

Research Advisor: Gonzalo M. Rivera
Director for Honors and Undergraduate Research: Sumana Datta

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ABSTRACT

The Role of Nck Adaptors in the Regulation of Endothelial Cell-Cell Adhesion.
(April 2011)

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The establishment of endothelial cell-cell contacts is critical for the development and maintenance of the vascular network. Endothelial cell adherens junctions (AJ) are cell-cell adhesion complexes consisting of clusters of vascular endothelial (VE) cadherin linked to the cortical actin cytoskeleton through catenins. Actin polymerization and remodeling are important for the formation, maturation, and turnover of AJ. Research was conducted to elucidate the role of the Nck family of SH2/SH3 domain-containing adaptor proteins, well established regulators of the actin cytoskeleton, in the formation and remodeling of AJ in endothelial cells. I tested the hypothesis that Nck adaptors contribute to the formation of a polarized vascular lumen through the enhancement of VE-cadherin-mediated cell-cell adhesions. The purpose of this study was to determine the role of Nck in the formation of VE-cadherin-based cell adhesions. To these aims, I used a combination of molecular genetics, cell biology, and high resolution fluorescent

microscopy. In the experiment, the effects of calcium were seen in the development of cell-cell junctions. When calcium was removed with EGTA, the VE-cadherin was delocalized from the edges of the cells. The EGTA also disrupted the actin fibers. The reintroduction of calcium reestablished the endothelial junctions between cells.

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CHAPTER I

INTRODUCTION

The establishment of endothelial cell-cell contacts promotes vascular morphogenesis and capillary formation. The formation of junctions could support intracellular signaling which would assist in cell growth, apoptosis, gene expression, and other cellular processes (Dejana, 2004). In the parts of the body where vascular permeability is strictly regulated, AJ are well organized and stable; however, in those locations where molecules and fluids are exchanged between the blood and tissues, these junctions are poorly organized. On the other hand, junctional integrity is characteristic of mature, stable vessels, whereas cell-cell junction disorganization and remodeling occurs during vascular morphogenesis. A better understanding of the molecular mechanisms controlling junctional organization of endothelial cells may open new avenues for the development of therapies to control vascular permeability in inflammatory diseases and target tumor angiogenesis.

The assembly and remodeling of AJ is linked to actin polymerization and reorganization. The development and maintainance of AJ is supported by microtubules and actin. AJ can also manage and organize mictrotubles and actin (Harris and Tepass, 2010) Sprouting angiogenesis is initiated by differentiated “tip” endothelial cells that respond to a gradient of angiogenic factors (such as vascular endothelial growth factor, VEGF) by

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This thesis follows the style of *Journal of Cell Biology.*

forming filopodia, finger-like membrane protrusions supported by parallel bundles of actin filaments (De Smet et al. 2009). Behind the tip endothelial cells, "stalk" endothelial cells elongate the nascent vessel by proliferation, the establishment of cell-cell contacts, and lumen formation. The assembly of cell-cell contacts is enabled by actin-based membrane protrusions that promote the formation of cadherin/catenin clusters, which in turn, stimulate further actin polymerization and expansion of the junctional structure (Harris and Tepass, 2010 and Almagro et al., 2010). Radial bundles of actin supporting AJ formation are replaced by a circumferential actin belt underlying stable AJ as the epithelial/endothelial layer matures. Two pools of F-actin appear to control the stability and mobility of E-cadherin clusters (Cavey et al., 2008); whereas small, relatively immobile actin patches stabilize E-cadherin clusters, a contractile, dynamic actin network maintains the adhesion in a defined domain by limiting its lateral mobility. In addition, tissue remodeling, including neovascularization, requires the disassembly of AJ and turnover of its components through endocytic/recycling pathways that require actin polymerization (Xiao et al., 2005 and de Beco et al., 2009).

The Nck family includes two closely related proteins, termed Nck1 (or α) and Nck2 (or β), that link extracellular signals with cytoskeletal remodeling (Li et al., 2001). These adaptors, consisting of three Src Homology (SH) 3 and one SH2 domain, are well suited to coupling signals mediated by tyrosine phosphorylation with cytoskeletal effectors. An increased local concentration of Nck induces N-WASp/Arp2/3-dependent actin polymerization in living cells (Rivera et al., 2009 and Rivera et al., 2004). These adaptors have been involved in vascular endothelial cell motility stimulated by the

activated VEGF receptor (VEGFR-2) (Stoletov et al., 2004 and Lamalice et al., 2006). It was recently shown that Nck adaptors are required for filopodia formation during cell attachment through a pathway that involves the tyrosine kinase Abl (Antoku et al., 2008). Actin reorganization induced by tyrosine kinases of the Abl family modulates cadherin-dependent cell-cell contacts (Zandy and Pendergast, 2008).

Modulation of actin dynamics by Nck adaptors may also play a role in the establishment of endothelial cell/vascular lumen polarity. For instance, Nck has been involved in EphA-ephrinA-dependent enhancement of E-cadherin-based cell-cell contacts and the establishment of apical-basal polarity in MDCK cells (Miura et al., 2009). Since EphA activation plays a critical role in VEGF-stimulated angiogenesis (Cheng et al., 2002), it is likely that Nck adaptors provide a physical link between these converging signaling pathways. Preliminary data strongly suggests the requirement of Nck in VEGF-stimulated vascular morphogenesis (Fig. 1).

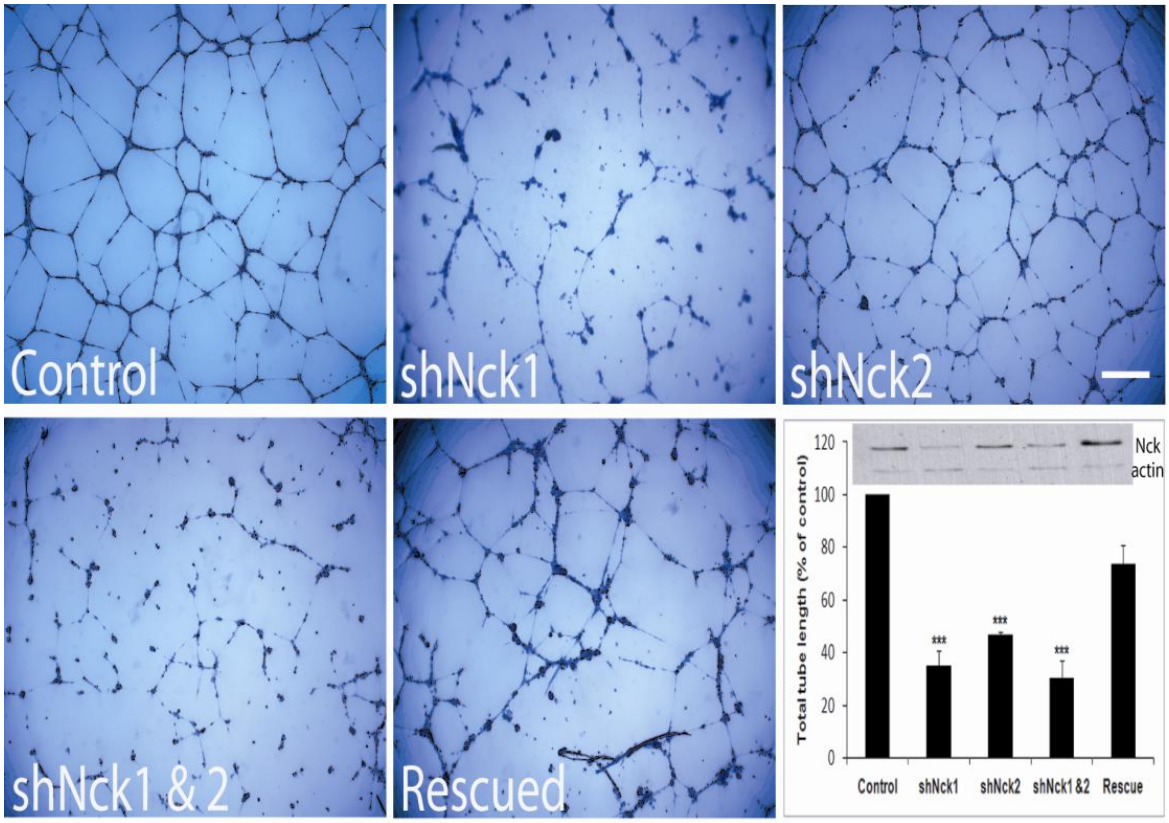

Fig. 1. Nck is required for VEGF-stimulated tube formation by endothelial cells. Normal human umbilical endothelial cells (control), cells with short hairpin (sh) RNA-mediated knockdown of Nck1 (shNck1), Nck2 (shNck2), double knockdown (shNck1 & 2), and double knockdown rescued with mouse Nck2 (Rescued) were seeded on an extracellular matrix (Matrigel®) and incubated overnight. Following incubation, cultures were fixed and stained to visualize tube formation. The lower panel on the right shows results of the quantitative analysis of tube length determined with MetaMorph®. Multiple images from 3 independent experiments were analyzed (*** $p < 0.05$). The western blot shows the levels of Nck corresponding to the different population of cells detailed in the bar graph.

CHAPTER II

METHODS

Tissue culture

Human umbilical vein endothelial cells (HUVEC) obtained from Lonza, Inc. (MD, USA) were used in the experiment. Cells were cultured in flasks/dishes pre-coated with fibronectin using endothelial cell basal medium (EBM-2, Lonza) supplemented with 2% fetal bovine serum, endothelial cell growth supplement (EGM-2, Lonza) in an atmosphere of 5% CO_2/95% air. When required, cells will be cultured under starvation conditions in EBM-2 supplemented with 0.2% fetal bovine serum. The cells were used on passage 6.

Plasmids and retroviral transduction for protein expression and protein knockdown

Stable expression of fluorescently-tagged proteins, including actin, and wild type or loss-of-function mutants of Nck and VE-cadherin were performed. Wild type Nck and Nck2, and VE-cadherin constructs subcloned into pMSCV-puro and p-MIGR retroviral vectors were already available. Short hairpin RNAs targeting proteins of interests were inserted into pSuper.retro vector (Oligoengine).

Ascertain the role of Nck in the distribution of AJ components, markers of cell polarity, and the structure of the actin cytoskeleton in endothelial cells.

The contribution of Nck in the remodeling of mature VE-cadherin-based cell-cell contacts was determined. Confluent monolayers of normal endothelial cells and endothelial cells with altered Nck signaling were starved overnight and fixed and subjected to a Ca2+ switch assay. Immunostaining of the AJ proteins VE-cadherin and β-catenin, as well as markers of endothelial cell polarity including podocalyxin and the basement membrane component collagen IV (basal marker) was performed with specific antibodies. The structure of the actin cytoskeleton was assessed by staining with fluorescent phalloidin. The procedure of the calcium switch assay was optimized after multiple experimentations.

For the calcium switch assay

On acid –washed coverslips, 650,000 cells were plated for confluent cells and 320,000 cells were plated for sub-confluent cells. It was determined that the time points best suited for the experiment were 15 minutes, 30 minutes, and 1 hour for the adding back of calcium. In each well, 2.5 mL of HUVEC Serum Starvation Media was added and the cell were kept overnight. For the wells that were to have calcium removed, 2mL of 4mM EGTA media was added and incubated for 30 minutes at 37 degrees Celsius. For the t=0 timepoint, the fixation step was followed; however, the other time points for the replacing of calcium received 2mL of starvation media with a calcium concentration of 1.8mM. In order to fix the cells, 800 ul of 3.7% PFA was added to each well and the

cells were incubated for 10 minutes at room temperature. The cells were then rinsed gently with 2mL of PBS once. Next, the cells were permeabilized with 200 ul of 0.25% TX-100 for 5 minutes at room temperature. The coverslips were then incubated with the primary antibody, VE-cadherin Rabbit, in 2% BSA (1:1000 dilution). One of the coverslips was incubated with normal rabbit IgG in 2% BSA(1:500 dilution) instead. On parafilm, 100 ul drops of the antibody/BSA were added and the coverslips were placed on top. Incubation took place for 1 hour at room temperature. The coverslips were then rinsed gently with 2mL of PBS four times for 3 minutes each on the belly dancer. The cells were then incubated with the secondary antibody, 488 Alexa in 2% BSA (1:1000 dilution) with DAPI and TX Red. The coverslips were placed on drops of secondary antibody on parafilm and incubated for 1 hour at room temperature. The coverslips were then rinsed gently with 2mL of PBS four times for 3 minutes each on the belly dancer. The coverslips were then mounted with Fluoromount G and were placed in a dark drawer overnight. The edges were then sealed with nail polish and were stored at 4 degrees Celsius.

Imaging

Confocal and epifluorescence imaging of living cells were performed with a Zeiss LSM 510 META NLO Confocal/multiphoton microscope and a Zeiss Stallion DDI digital imaging system. Imaging equipment, software for image analysis, and technical assistance were available through the TAMU Image Analysis Laboratory. Quantitative, computer-assisted image analysis was performed with ImageJ using a computer

algorithm specifically designed to quantify localized actin polymerization and aggregates of fluorescent proteins from confocal images.

CHAPTER III

RESULTS

Multiple experiments using differing conditions were performed in order to determine the effect of Nck adaptors in cell-cell adhesion with HUVEC cells. The Western Blot shows the effectiveness of the antibodies (Figure 2). For the first experiment (Figure 3), HUVEC cells were plated into two 6-well plates for viral infections with a cell density of 10x10^4 cells/well. Before the immunofluorescence experiment, large fibronectin coverslips were used at the bottoms of each well. In order to improve some of the images, three changes were made to the experiment. In the second experiment, all conditions were kept the same except that the 6 well plate was put on ice when adding TX-100, PBS was used with the primary antibody instead of blocking solution, and secondary wavelength of 488 was used for imaging instead of 647. The images showed room for areas of improvement due to the cells being concentrated at the center of the coverslips. A weaker signal was given for multiple images. In the third draft of the experiment, the cell count was increased to 60x10^4 cells/well. The effects of using PBS versus cytoskeletal buffer were also tested. The PBS gave clearer and more precise images than the other buffer. In the next draft of the experiment, small coverslips were used instead of larger. The images showed areas of darkness around the edges of the slide. The cause of this was that the smaller coverslips sat more on top of the solution in the well instead of at the bottom. This caused the cells to be viewed on different planes and not all were able to be captured in the imaging. Imaging was taken using Z-stacks in

order to get a better picture, but the experiment was again repeated with large coverslips. Two different sets of cells were used with the large coverslips, one set was subconfluent (Figure 4A) while the other was confluent (Figure 4B). Collectively, in all of the images there was a clear difference between the control cells and the knockdown group. With the cells treated with EGTA, the VE-cadherin was not localized around the edges of the cells and was more concentrated in the cytosol. After calcium was added back to the cells, the VE-cadherin becomes increasingly more concentrated around the cell edges with each time point. The EGTA also seemed to have an effect on the actin, which did not look as defined and filamentous.

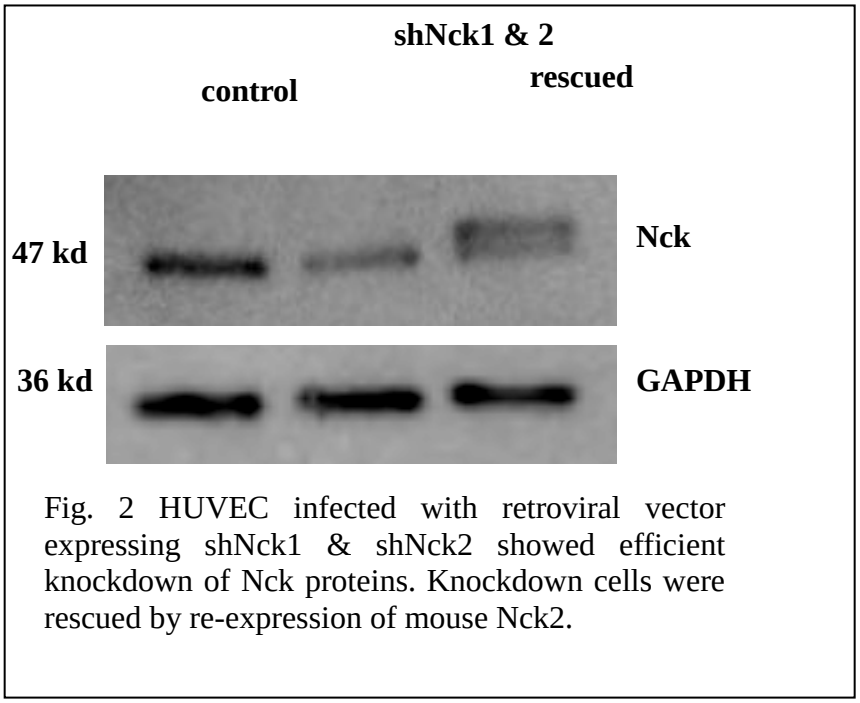

Fig. 2 HUVEC infected with retroviral vector expressing shNck1 & shNck2 showed efficient knockdown of Nck proteins. Knockdown cells were rescued by re-expression of mouse Nck2.

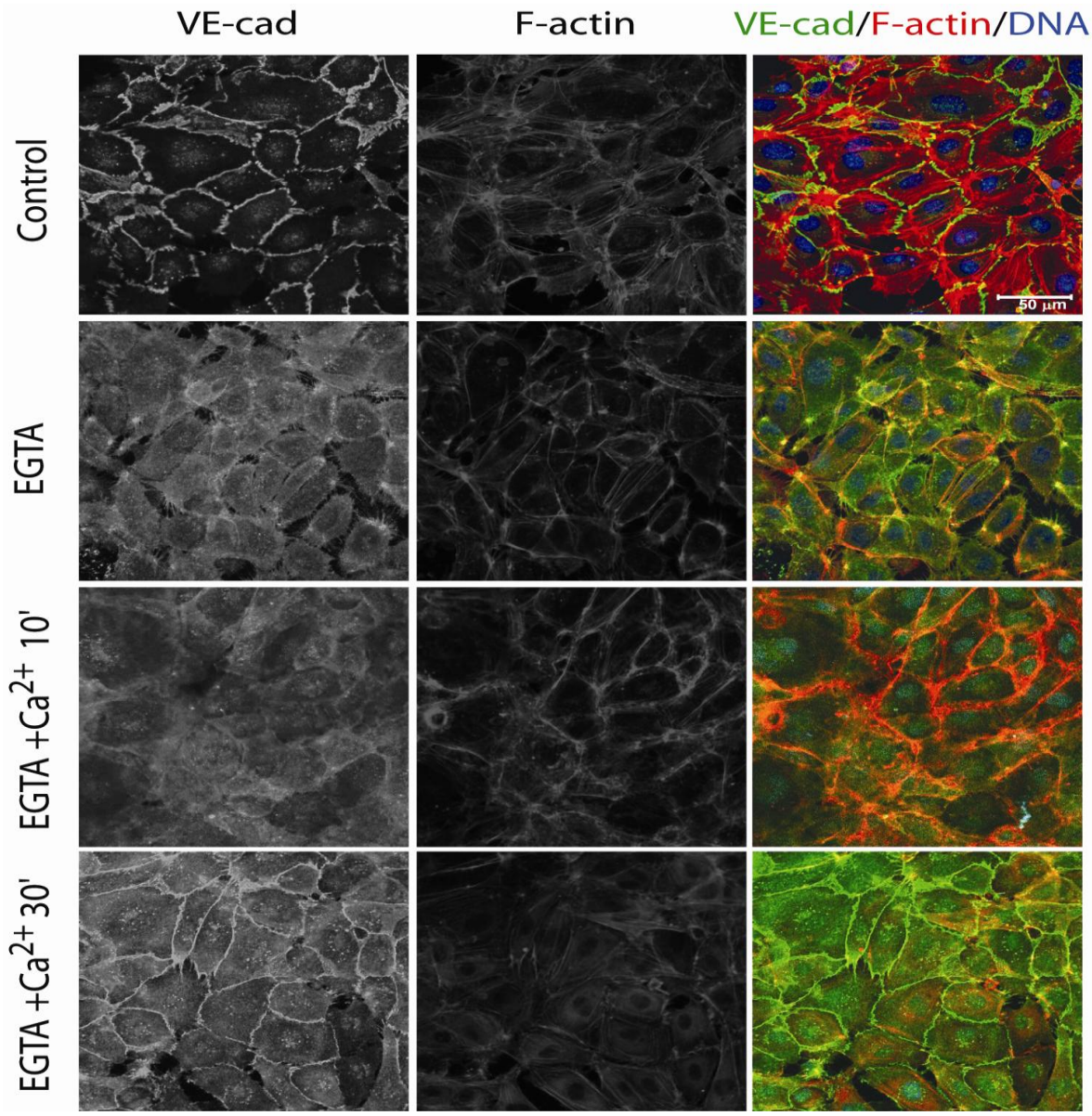

Fig. 3. Endothelial cell-cell contacts require calcium-dependent VE-cadherin interactions. Confocal images of human umbilical vein endothelial cells showing the distribution of endogenous VE-cadherin and the architecture of the actin cytoskeleton. Cells were serum-starved overnight and fixed (control), or incubated for 30 minutes in the presence of 4 mM EGTA to deplete calcium. EGTA-treated cells were immediately fixed (EGTA) or re-exposed to calcium-containing medium for 10 (EGTA+Ca^{++}-10') or 30 (EGTA+Ca^{++}-30') minutes. Cells were subjected to immunofluorescence with a rabbit polyclonal anti-VE-cadherin antibody, and filamentous actin and nuclei were stained with fluorescent phalloidin and DAPI, respectively. Well organized cell-cell junctions (control) are disrupted by calcium depletion (EGTA) and re-established by calcium replenishment (EGTA+Ca^{++}-10' and EGTA+Ca^{++}-30'). Calcium depletion also induces cytoskeletal rearrangements, including the partial disassembly actin stress fibers. Bar represents 50 µM.

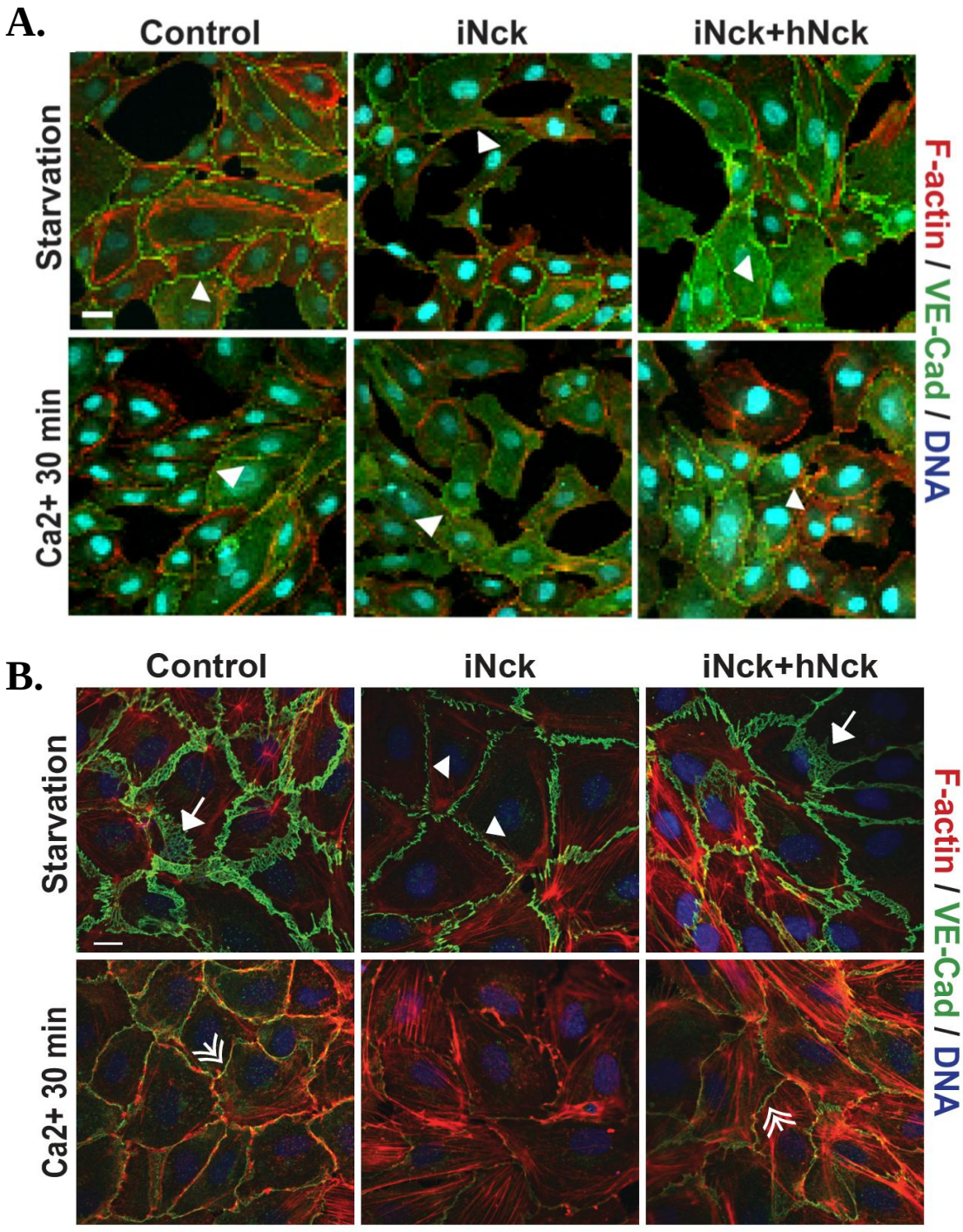

Fig. 4. A. Sub-confluent & B. Confluent: Endothelial cell-cell contacts require calcium-dependent VE-cadherin interactions. Confocal images of human umbilical vein endothelial cells showing the distribution of endogenous VE-cadherin and the architecture of the actin cytoskeleton. Cells were serum-starved overnight and fixed (control), or incubated for 30 minutes in the presence of 4 mM EGTA to deplete calcium. EGTA-treated cells were immediately fixed (EGTA) or re-exposed to calcium-containing medium for 30 (EGTA+Ca^{++}-30') minutes. Cells were subjected to immunofluorescence with a rabbit polyclonal anti-VE-cadherin antibody, and filamentous actin and nuclei were stained with fluorescent TX-Red and DAPI, respectively. Well organized cell-cell junctions (control) are disrupted by calcium depletion (EGTA) and re-established by calcium replenishment (EGTA+Ca^{++}-30'). Calcium depletion also induces cytoskeletal rearrangements, including the partial disassembly actin stress fibers. Bar represents 50 µM.

CHAPTER IV

SUMMARY AND CONCLUSIONS

With the use of high microscopy imaging, the adhering junctions were able to be observed for three different HUVEC cell conditions including controls, Nck KD, and rescues. After performing the calcium switch experiment in which some cells were given EGTA and others were not, the effects of calcium were observed in the formation of these cell-cell junctions. Those cells with a thirty minute EGTA treatment showed delocalization of VE-cadherin from the edges of the cell. Adherens junctions were not present between the cells with this condition and a decrease in cell communication was assumed. Also, the actin seemed to be effected by the EGTA treatment and was not as filamentous as in the control starvation cells. Three different time points were chosen to apply calcium back to the EGTA treated cells. The fifteen minute calcium treated cells showed a slight sign of relocalization of VE-cadherin and junctions were starting to reform between adjacent cells; however, this appearance was minimal compared to the original adherens junctions. After thirty minutes, an increase in relocalization was seen, but less was observed in comparison to the one hour calcium treatment. With the one hour treatment, the VE-cadherin was relocalized around the cell edges and was no longer concentrated in the cytosol of the HUVEC cells.

When comparing the control cells to the Nck knockdown group, it is clear that Nck plays a role in the formation of cell-cell adhesions. The VE-cadherin was not as pronounced as

in the control cell group. The rescue cells showed an increased signal of cellular junctions in comparison to the knockdowns; however, only a small difference was noticed. In future experiments, multiple western blots should be performed in order to ensure that the antibodies are effective. Also, a larger time point may allow more time for the cellular junctions to reestablish more effectively.

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